BIOACTIVE OXYLIPINS FROM THE ENDOPHYTE *KHUSKIA ORYZAE* ISOLATED FROM THE MEDICINAL PLANT *BIDENS ALBA*

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ABSTRACT

An endophytic fungus was isolated from the medicinal plant *Bidens alba* and was identified as *Khuskia oryzae* on the basis of ITS sequence comparison. The ethyl acetate extract of the fungal isolate obtained exhibits antimicrobial activity in agar diffusion assays as well as cytotoxic and antiproliferative effects. Activity-guided chromatographic fractionation resulted in the isolation of the active metabolites which were identified by different spectroscopic techniques as the bioactive oxylipin called 8-oxo-(9E, 11E)-octadecadienoic acid and the oxygenated bioactive fatty acid 9-oxo-(10E, 12E)-octadecadienoic acid. A cytotoxic assay was performed for the compounds and revealed the first to be of cytotoxic activity against HeLa cell lines with a CC_{50} value of 42.6 µg mL^{-1} and cytostatic activity against HUVEC and K-562 cancer cell lines with GI_{50} values of 40.7 µg mL^{-1} and 41.5 µg mL^{-1} respectively.

The cytotoxic assay performed on 8-oxo-(9E, 11E) octadecadienoic acid showed cytotoxicity against HeLa cell lines with a CC_{50} value of 33.3 µg mL^{-1} and cytostatic activity against HUVEC and K-562 cell lines with a GI_{50} value of 30.8 µg mL^{-1} and 28.8 µg mL^{-1} respectively.

In the antimicrobial assay performed against *B. subtilis* and *A. terreus* weak antibacterial and antifungal activities were observed for 9-oxo-(10E, 12E)-octadecadienoic acid (inhibition

zone 16 mm and 12 mm at a conc. of 250 µg mL^{-1} respectively), while moderate antibacterial and antifungal activities were detected for 8-oxo-(9E, 11E)-octadecadienoic acid (inhibition zone 20 mm and 16 mm at a conc of 250 µg mL^{-1}respectively).

KEYWORDS: oxylipin, endophyte, *Khuskia oryzae, Bidens alba.*

INTRODUCTION

Microorganisms represent a promising rich source of novel natural product leads having the advantage of feasible production of large quantities with reasonable cost, by large scale cultivation and fermentation of the source organisms [1]. Although it appears that the pharmaceutical industry has completely exhausted the hidden treasures of the microbial world to find solutions for the infectious diseases of the last century, a report by the American Academy of Microbiology estimates that less than 5% of fungal species are currently known and only 1% of these microbes have been cultured and characterized. Thus challenges to find novel microbes remain. Endophytes have been identified as a promising source of new pharmacologically active secondary metabolites that might be suitable for medicinal or agrochemical applications. Several antimicrobial agents have been discovered from microbial sources since the discovery of penicillin G in 1928 [2]. Cephalosporin C is one of the famous antibacterial agents discovered after penicillin G [3], while griseofulvin is one of the first antifungal agents obtained from fungi [4].

Bidens alba is an annual herb found in tropical and subtropical regions of the world [5]. It is distributed in Africa, America, China, and Japan that is used in traditional medicines for treatment of inflammation, hepatitis and diabetes [6]. In addition, extracts from *B. alba* contain flavonoids that exert in vitro antimalarial activity against *Plasmodium falciparum* [7].

MATERIALS AND METHODS

Strain isolation and taxonomic classification

Samples of *B. alba* were collected near Cairo, Egypt. After surface sterilization of the fresh, healthy, aerial plant parts an endophytic fungal strain was isolated. The strain was cultivated in four different culture media a malt extract (M4), a caseine–flesh peptone (M5), a cornsteep (M25) and a dextrose–yeast (M26) medium both as a shaken and as a stationary culture and was then subjected to antimicrobial activity screening. Results of the agar diffusion assay performed showed moderate antibacterial activity against *Bacillus subtilis* as well as moderate antifungal activity against *A. terreus.*

The strain was identified as *Khuskia oryzea* by ITS sequence comparison. Literature data showed that the only known metabolite of this rarely investigated strain was the antifungal agent griseofulvin [8].

Endophyte fermentation, extraction and isolation

Large scale fermentation (30 L) was performed in medium M25, where it showed the highest antimicrobial activity. Total extraction of culture broth and mycelium with ethyl acetate yielding 7 g of dried crude extract after solvent evaporation was carried out. Chromatographic fractionation of the extract was performed on silica gel using a solvent system of hexane: ethyl acetate starting with a proportion of 9:1 and then gradually increasing the proportion of ethyl acetate till final elution with 100% ethyl acetate. After combining the similar fractions three main fractions were obtained. Activity guided fractionation resulted in the isolation of three pure compounds after several purification steps on Sephadex LH-20 using methanol as a solvent and finally isolation of the pure compounds using RP silica on the preparative HPLC.

General

NMR spectra were recorded on a Bruker DPX-300 and a Bruker DRX-500 at 300 MHz and 500 MHz for ^{1}H, and 125 MHz for ^{13}C NMR, respectively; chemical shifts are given in δ values (ppm) and were measured relative to tetramethylsilane as standard. IR spectra were recorded on a Bruker FT-IR (IFS 55) spectrometer. UV spectra were recorded on a Cary 1 Bio UV vis spectrophotometer (Variant). HPLC-MS measurements were recorded on an Agilent high performance 1100 series LC/MSD Trap module with an API - electrospray source, PC printer and LC/MSD chemstation software for data acquisition and data analysis. HRESIMS were recorded on a Finnigan TSQ Quantum Ultra AM Thermo Electron. Open column chromatography was performed on silica gel 60 (Merck, 0.04-0.063 mm, 230-400 mesh ASTM) and Sephadex LH-20 (Pharmacia). TLC: silica gel plates (silica gel 60 F_{254} on aluminum foil or glass, Merck), spots were visualized by spraying with anisaldehyde/sulfuric acid followed by heating. Analytical HPLC was conducted on a Shimadzu HPLC system using a Nucleosil 100-5 C_{18} column (5 µm, 125 x 4.6 mm) with MeCN/0.1% TFA-H_2O as eluent (flow rate 1 mL/min, 15/85 to 100% MeCN in 30 min) and UV detection at 254 nm. Preparative HPLC was performed on a Shimadzu HPLC system using a Nucleosil 100-5 C_{18} column (5 µm, 250 x 16 mm, pore diameter 100 Å) using a flow rate of 10 mL/min starting

elution with 25% MeCN and ending with 100% MeCN in 45 min with a UV detector. All solvents used were spectral grade or distilled prior to use.

Antimicrobial assay

Antifungal activities were studied qualitatively by agar diffusion tests according to the literature [9, 10].

Antiproliferative and cytotoxic assays

Cells and culture conditions

Cells of HUVEC (ATCC CRL-1730), K-562 (DSM ACC 10) and HeLa (DSM ACC 57) were cultured in DMEM (CAMBREX 12-614F), RPMI 1640 (CAMBREX 12-167F) and RPMI 1640 (CAMBREX 12-167F) respectively. All cells were grown in the apropriate cell culture medium supplemented with 10 mL/L ultraglutamine 1 (Cambrex 17-605E/U1), 500 µL/L gentamicin sulfate (CAMBREX 17-518Z), and 10% heat inactivated fetal bovine serum (PAA A15-144) at 37°C in high density polyethylene flasks (NUNC 156340).

Antiproliferative assay

The assay was carried out according to previously described method (Dolezal, 2009). The test substances were dissolved in DMSO before being diluted in DMEM. The adherent cells were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L 2163). For each experiment, approximately 10 000 cells were seeded with 0.1 mL culture medium per well of the 96-well microplates (NUNC 167008).

Cytotoxic assay

For the cytotoxic assay, HeLa cells were pre-incubated for 48 h without the test substances. The dilutions of the compounds were carried out carefully on the subconfluent monolayers of HeLa cells after the pre-incubation time. Cells were incubated with dilutions of the test substances for 72 h at 37°C in a humidified atmosphere and 5% CO_2.

Method of evaluation

For estimating the influence of chemical compounds on cell proliferation of K-562, the numbers of viable cells present in multiwall plates were determined via CellTiter-Blue® assay. The indicator dye resazurin was used to measure the metabolic capacity of cells as an indicator of cell viability. Viable cells of untreated control retain the ability to reduce

resazurin into resorufin, which is highly fluorescent. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal. Under our experimental conditions, the signal from the CellTiter-Blue® reagent is proportional to the number of viable cells. The adherent HUVEC and HeLa cells were fixed by glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gentle washing the stain was eluted with 0.2 mL of 0.33 N HCl in the wells. The optical densities were measured at 660 nm in SUNRISE microplate reader (TECAN). The GI_{50} and CC_{50} values were defined as being where the dose response curve intersected the 50% line, compared to untreated control. The comparisons of the different values were performed with software Magellan (TECAN).

RESULTS

A molecular formula of $C_{18}H_{31}O_3$ (*m/z* 295.2056 [M+H]$^+$) was suggested by HRESIMS for the first isolated compound thus indicating four degrees of unsaturation. The ^{13}C NMR spectrum revealed the presence of 18 carbons two of which represented a carbonyl and a carboxyl group, respectively. The presence of two olefinic bonds was deduced from the appearance of signals at chemical shift values of δ 128.07, 142.78, 128.97, and 145.36 ppm in the ^{13}C NMR spectrum. The pattern of ^1H NMR chemical shifts of the four olefinic protons at δ 6.1 ppm (d, *J* = 15.5 *Hz*), 6.2 ppm (d, *J* = 15.5 *Hz*), 6.3 ppm (d, *J* = 15.5 *Hz*), 7.2 ppm (dd, *J* = 2.8, 8.8, 15.5 *Hz*) suggested the presence of a diene system in the compound and the coupling constants indicated a *trans* configuration of the olefinic protons being thus in clear agreement with a 10*E*, 12*E* diene system. The HMBC experiment showed correlations between H-2 and C-1, C-5 and C-6 as well as between H-10 and C-9 and C-12. Furthermore HMBC correlations were observed between H-11 and C-13 as well as between H-12 and C-11 which served to establish the structure as 9-oxo-(10*E*, 12*E*)-octadecadienoic acid (Fig 1).
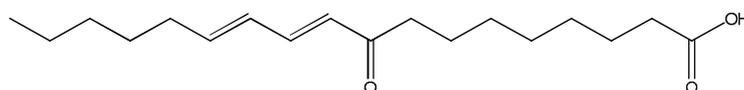

Fig 1. Chemical structure of 9-oxo-(10*E*, 12*E*) octadecadienoic acid

For the second compound a molecular formula of $C_{17}H_{29}O_3$ (as *m/z* 281.1055 [M+H]$^+$) was deduced from HRESIMS, indicating four degrees of unsaturation. The ^{13}C NMR spectrum revealed the presence of 17 carbon signals with one representing a carbonyl, a carboxyl and four sp^2 carbons. The NMR data (Table 1) of the compound showed great similarity to those of 9-oxo-(10*E*, 12*E*)-octadecadienoic acid, the only difference observed was the absence of

one methylenic carbon signal. The presence of a diene system in the structure was supported by the appearance of four olefinic protons in the ^{1}H NMR spectrum. The pattern of ^{1}H NMR chemical shift values of the four olefinic protons and their coupling constants were also characteristic for a 9*E*, 11*E* diene system. The structure of the compound (Fig 2) was established by detailed 2D NMR spectroscopic studies including COSY, HMQC and HMBC experiments (Fig 3), which revealed the compound as a new oxylipin called 8-oxo-(9*E*, 11*E*)-octadecadienoic acid.
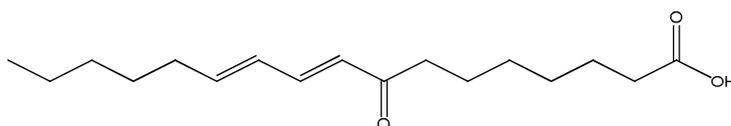
Fig 2. Chemical structure of 8-oxo-(9*E*, 11*E*)-octadecadienoic acid

Table 1. NMR data (125 MHz, DMSO-d$_6$) of 8-oxo-(9*E*, 11*E*)-octadecadienoic acid

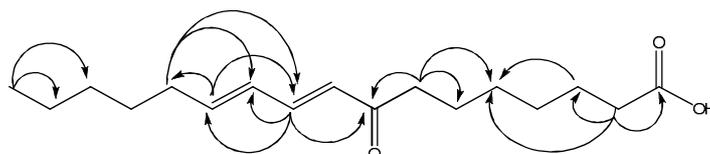| Position	δ^{13}C	δ^{1}H (*J* in *Hz*)	HMBC
1	174.9, qC		
2	33.8, CH$_2$	2.15, *m*	1, 3, 5
3	24.5, CH$_2$	1.45, *m*	5
4	28.0, CH$_2$	1.35, *m*	
5	28.5, CH$_2$	1.20, *m*	
6	23.9, CH$_2$	1.40, *m*	
7	40.0, CH$_2$	2.55, *m*	5, 6, 8
8	200.7, qC		
9	128.2, CH	6.10, *d* (15.5)	
10	143.2, CH	7.15, *dd* (2.7; 8.9; 15.5)	8, 11, 12
11	129.2, CH	6.26, *d* (15.5)	13
12	145.8, CH	6.25, *d* (15.5)	10, 13
13	32.6, CH$_2$	2.12, *m*	10, 11
14	28.5, CH$_2$	1.25, *m*	
15	31.0, CH$_2$	1.22, *m*	
16	22.1, CH$_2$	1.25, *m*	
17	14.1, CH$_3$	0.85, *m*	15, 16

Fig 3. Key HMBC correlations of 8-oxo-(9*E*, 11*E*)-octadecadienoic acid

A cytotoxic assay was performed for both compounds and revealed the first to be of cytotoxic activity against HeLa cell lines with a CC_{50} value of 42.6 µg mL^{-1} and cytostatic activity against HUVEC and K-562 cancer cell lines with GI_{50} values of 40.7µg mL^{-1} and 41.5 µg mL^{-1} respectively (Fig 4).

The cytotoxic assay performed on 8-oxo-(9*E*, 11*E*) octadecadienoic acid showed cytotoxicity against HeLa cell lines and also cytostatic activity against HUVEC and K-562 cell lines with a CC_{50} value of 33.3 µg mL^{-1} for the cytotoxic activity and a GI_{50} value of 30.8 µg mL^{-1} and 28.8 µg mL^{-1} forr the cytostatic activity against HUVEC and K-562 cell lines respectively (Fig 4). Compared to the first oxylipin isolated from this strain 8-oxo-(9*E*, 11*E*)-octadecadienoic acid exerts higher cytostatic and cytotoxic activities. In the antimicrobial assay performed against *B. subtilis* and *A. terreus* moderate antibacterial activity was observed for 9-oxo-(10*E*, 12*E*)-octadecadienoic acid (inhibition zone 16 mm at a conc. of 250 µg mL^{-1}) and also for 8-oxo-(9*E*, 11*E*) octadecadienoic acid (inhibition zone 20 mm at a conc. of 250 µg mL^{-1}). Weak antifungal activity against *A. terreus* was observed for 9-oxo-(10*E*, 12*E*)-octadecadienoic acid (inhibition zone 12 mm at a conc. of 250 µg mL^{-1}) and moderate activity was exerted by 8-oxo-(9*E*, 11*E*)-octadecadienoic acid (inhibition zone 16 mm at a conc. of 250 µg mL^{-1}) against the same microorganism.
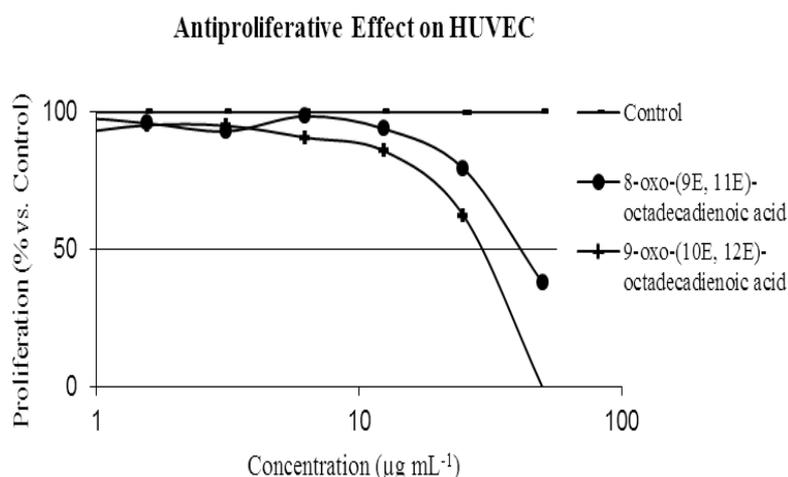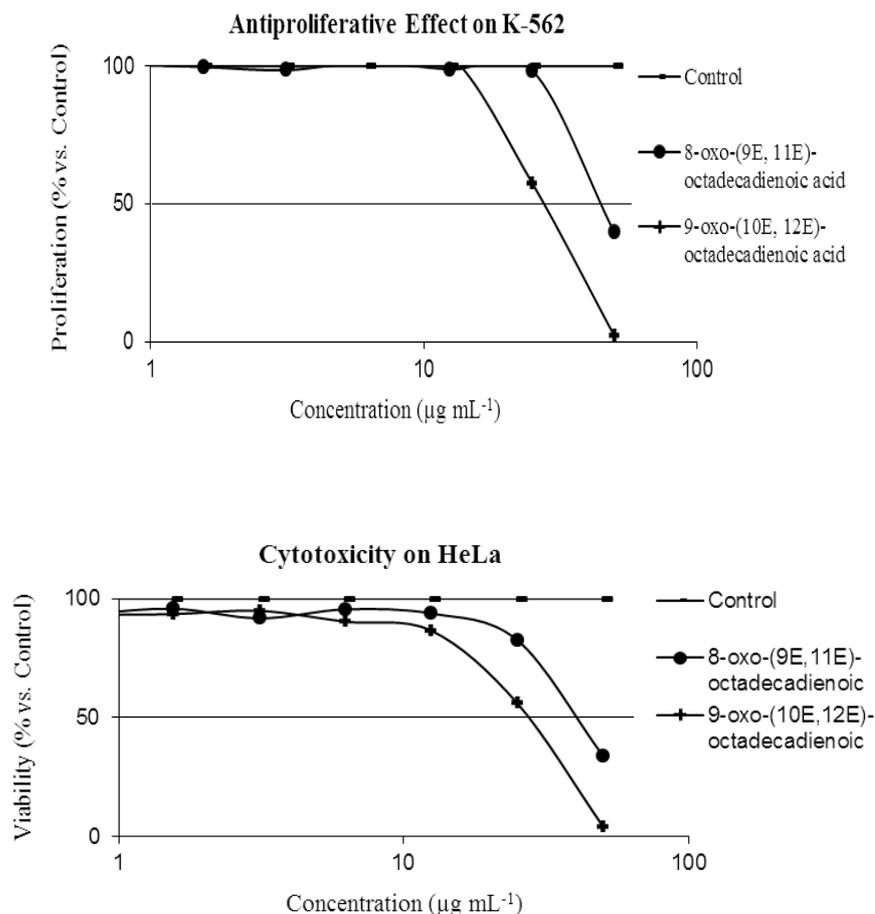
Fig 3. Antiproliferative and cytotoxic activities of 9-oxo-(10*E*, 12*E*)-octadecadienoic acid and 8-oxo-(9*E*, 11*E*)-octadecadienoic acid against HUVEC, K-562 and HeLa cell line

DISCUSSION

The oxygenated derivatives of fatty acids, known as oxylipins to which the two isolated secondary metabolites of *K. oryzae* belong, are important signaling molecules in animals and terrestrial plants [16]. In animal systems eicosanoids regulate cell differentiation, immune response and homeostasis. In contrast terrestrial plants use derivatives of C18 and C16 fatty acids as developmental or defense hormones. The oxylipin 9-oxo-(10*E*, 12*E)* octadecadienoic acid was previously found in the marine red alga *Chondrus crispus* and was found to be involved in induction of innate immunity of this alga [16]. Furthermore it was found in another study to have good activity against the bacterial plant pathogens *Phytophthora parasitica* and *Cladosprium herbarum* which is why it was suggested to contribute to plant protection not only by induction of defensive responses but also by direct antimicrobial activity in some cases [17]. More recently a study was conducted on the fungitoxic constituents of the

basidiomycete *Gomphus floccosus* and detected 9-oxo-(10*E*, 12*E*)-octadecadienoic acids together with two other oxylipins as the active constituents with predominant antifungal activity against *Phomopsis* species [18].

CONCLUSION

In conclusion, a fungal endophyte has been isolated from the important plant *B. alba* and was identified as a *K. oryzae* strain. Through bioactivity-guided fractionation the isolation and full characterization of two oxylipins which have rarely been isolated from this fungal species before was accomplished.

ACKNOWLEDGEMENTS

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